

**METHOD FOR IDENTIFYING SUBSTANCES WHICH POSITIVELY  
INFLUENCE INFLAMMATORY CONDITIONS OF CHRONIC  
INFLAMMATORY AIRWAY DISEASES**

**Related Application**

The benefit of prior provisional application Serial No. 60/233,748, filed September 19, 2000 is hereby claimed.

**Background of the Invention**

The present invention belongs to the field of modulation of inflammatory processes, in particular of inflammatory airway diseases, in which macrophages play an important role. The inflammatory processes can be modulated according to the invention by influencing the function of receptors on macrophages, which receptors are identified to be involved in the inflammatory process.

Inflammatory processes involve a cascade of reactions. A wide variety of factors are involved in inflammatory processes leaving a single treatment to avoid said factors unsuccessful. This is in particular true for inflammatory processes of the airways, like the chronic inflammatory airway diseases.

Chronic inflammatory airway diseases include Chronic Bronchitis and Chronic Obstructive Pulmonary Disease (COPD). For example, COPD is a complex disease encompassing symptoms of several disorders: chronic bronchitis which is characterized by cough and mucus hypersecretion, small airway disease, including inflammation and peribronchial fibrosis, and emphysema. COPD is characterized by an accelerated and irreversible decline of lung function. The major risk factor for developing COPD is continuous cigarette smoking. Since only about 20% of all smokers are inflicted with COPD, a genetic predisposition is also likely to contribute to the disease.

The initial events in the early onset of COPD are inflammatory, affecting small and large airways. An irritation caused by cigarette smoking attracts macrophages and neutrophils the number of which is increased in the sputum of smokers. Perpetual smoking leads to an ongoing inflammatory response in the lung by releasing mediators from macrophages, neutrophils and epithelial cells that recruit inflammatory cells to sites of the injury. So far

there is no therapy available to reverse the course of COPD. Smoking cessation may reduce the decline of lung function. Only a few drugs provide some relief for patients. Longlasting  $\beta_2$ -agonists and anticholinergics are applied to achieve a transient bronchodilatation. A variety of antagonists for inflammatory events are under investigation like,  $LTB_4$ -, IL-8-,  $TNF\alpha$ - inhibitors.

Chronic inflammatory airway diseases can be attributed to activated inflammatory immune cells, e.g. macrophages. There is a need for modulating the function of macrophages in order to eliminate a basis for inflammatory processes.

### **Summary of the Invention**

The present invention relates to substances which modulate receptors involved in inflammatory processes and whose modulated functions positively influence inflammatory diseases.

### **Description of the Invention**

In the present invention it was found that macrophages involved in an inflammatory process, preferably in a chronic inflammatory airway disease, more preferably in chronic bronchitis or COPD, show a pattern of differentially expressed nucleic acid sequence and protein expression which differs from the pattern of gene expression of macrophages from healthy donors or donors in an irritated status, which latter do contain macrophages in an activated status. Therefore, macrophages show different activation levels under different inflammatory conditions, and it is shown in the present invention that macrophages in a hyperactive status are involved in an inflammatory process, preferably in a chronic inflammatory airway disease, more preferably in chronic bronchitis or COPD. The present invention provides for the inhibition of the hyperactivation or the reduction of the hyperactive status of a macrophage by allowing the identification of substances which modulate receptors involved in the hyperactivation or maintaining the hyperactive status.

The invention is based on the identification of a differentially expressed nucleic acid sequence or protein which is involved in causing the induction and/or maintenance of the hyperactive status of macrophages involved in an inflammatory process, preferably in a chronic inflammatory airway disease, more preferably in chronic bronchitis or COPD. Such differentially expressed nucleic acid sequence or protein is in the following named

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differentially expressed nucleic acid sequence or protein of the invention respectively. In particular, the present invention teaches a link between phenotypic changes in macrophages due to differentially expressed nucleic acid sequence and protein expression pattern and involvement of macrophages in inflammatory processes and, thus, provides a basis for a variety of applications. For example, the present invention provides a method and a test system for determining the expression level of a macrophage protein or differentially expressed nucleic acid sequence of the invention and thereby provides e.g. for methods for diagnosis or monitoring of inflammatory processes with involvement of hyperactivated macrophages in mammalian, preferably human beings, especially such beings suffering from an inflammatory process, preferably in a chronic inflammatory airway disease, more preferably in chronic bronchitis or COPD. The invention also relates to a method for identifying a substance by means of a differentially expressed nucleic acid sequence or protein of the invention processes, which substance modulates, i.e. acts as an inhibitor or activator on the said differentially expressed nucleic acid sequence or protein of the invention and thereby positively influences chronic inflammatory processes by inhibition of the hyperactivation or reduction of the hyperactive status of macrophages, and thereby allows treatment of mammals, preferably human beings, suffering from a said disease. The invention also relates to a method for selectively modulating such a differentially expressed nucleic acid sequence or protein of the invention in a macrophage comprising administering a substance determined to be a modulator of said protein or differentially expressed nucleic acid sequence. The present invention includes the use of said substances for treating beings in need of a treatment of an inflammatory process, preferably a chronic inflammatory airway disease, more preferably chronic bronchitis or COPD.

For the present invention in a first step differentially expressed nucleic acid sequences and proteins are identified which have a different expression pattern in a hyperactivated macrophage compared to a macrophage which is not hyperactivated. For the sake of conciseness this description deals particularly with investigation of macrophages involved in COPD, however, equivalent results may be observed with samples from patients suffering from other chronic inflammatory airway diseases, e.g. chronic bronchitis. The investigation of the different expression pattern leads to the identification of a series of differentially expressed nucleic acid sequences in macrophages, differentially expressed in dependency on the activation status of a macrophage involved in an inflammatory process, as exemplified in the Examples hereinbelow.

Briefly, such a differentially expressed nucleic acid sequence is identified by comparative expression profiling experiments using a cell or cellular extract from a hyperactivated macrophage, i.e. for example from the site of inflammation in a COPD and from the corresponding site of control being not suffering from said disease, however, suffering from an irritated condition like cigarette smoke exposure.

A differentially expressed nucleic acid sequence or protein of the invention can easily be detected by such a method because amongst the differentially expressed macrophage genes a class of differentially expressed nucleic acid sequences can be identified which encodes a class of macrophage surface receptors which is characterized in that it is expressed at a lower or higher level than the control level in a macrophage which is not hyperactivated. Such a macrophage surface receptor of the invention is hereinafter named ILM receptor. However, the invention does not only concern a naturally occurring ILM receptor, but also includes within the meaning of ILM receptor a receptor which is functionally equivalent to, i.e. which shares the binding capacities and the cellular function with an ILM receptor.

An example for an ILM receptor according to the present invention is a FPRL-1 receptor type receptor including FPRL-1 receptor (SEQ ID NO:2). The term "receptor type receptor" used in context with the present invention, e.g. FPRL-1 receptor type receptor, is a receptor which is "functionally equivalent" to, i.e. which shares the binding capacities and the cellular function with, the respective receptor, e.g. FPRL-1 receptor of SEQ. ID NO:2; the term also encompasses variants, mutants or fragments of a naturally occurring receptor, e.g. FPRL-1 receptor. or naturally occurring receptor type receptor, e.g. FPRL-1 receptor type receptor, which variants, mutants or fragments are functionally equivalent to the receptor, e.g. FPRL-1 receptor.

Further examples for ILM receptors are HM74 receptor type receptor including HM74 receptor (SEQ ID NO:21); AICL receptor type receptor including AICL receptor (SEQ ID NO:6); ILT1 receptor type receptor including ILT1 receptor (SEQ ID NO:12); SHPS-1 receptor type receptor including SHPS-1 receptor (SEQ ID NO:4); KDEL receptor 1 type receptor including KDEL receptor 1 (SEQ ID NO:8); and CSF-1 receptor type receptor including CSF-1 receptor (SEQ ID NO:10). Preferred is the respective receptor shown in the sequence listing or a variant, mutant or fragment thereof having the same function, even more preferred is the respective receptor shown in the sequence listing under SEQ ID NOs:21, 6, 12, 4, 8, 10. In even more preferred embodiments the receptors are encoded by the nucleic acid sequences having the SEQ ID NOs:20, 5, 11, 3, 7 or 9, respectively.

A preferred embodiment of an ILM receptor in context with the present invention is a FPRL-1 receptor type receptor. The term FPRL-1 receptor type receptor accordingly also encompasses variants, mutants or fragments, of naturally occurring FPRL-1 receptor or FPRL-1 receptor type receptors, which variants, mutants or fragments are functionally equivalent to the FPRL-1 receptor. An even more preferred embodiment in context with the description of the embodiments of the present invention is the FPRL-1 receptor of SEQ ID NO:2 or a variant, mutant or fragment thereof having the same function, even more preferred is the FPRL-1 receptor of SEQ ID NO:2. In a most preferred embodiment, the FPRL-1 receptor is encoded by the nucleic acid sequence shown in SEQ ID NO:1.

According to the present invention, the function of an ILM receptor expressed at a lower level than the control level is preferably activated in order to inhibit hyperactivation or reduce a hyperactivated status of a macrophage, whereby the function of an ILM receptor which is expressed at a higher level than the control level is preferably inhibited in order to inhibit hyperactivation or reduce a hyperactivated status of a macrophage. A function of a receptor in context with the present invention is any function of a receptor of the invention which is capable of influencing the inflammatory processes. For example, a receptor of the invention mediates inflammation in that it is activated by a ligand (any substance which has the capacity to bind to said receptor to at least one of its domains exposed on the cell surface) and leads to an intracellular signal involved in inflammatory processes.

In one embodiment the present invention concerns a method for determining a substance to be an activator or inhibitor of an ILM receptor characterized in that the receptor is deregulated preferably overexpressed or downregulated in a macrophage involved in a chronic inflammatory airway disease and which receptor plays a role in mediating inflammation. A method according to the invention comprises the application of a substance of interest to a test system which generates a measurable read-out upon modulation of the ILM receptor or of an ILM receptor function. A test system useful for performing such method of the invention comprises a cell or a cell-free system. For example, in one embodiment according to the invention the system is designed in order to allow the testing of substances acting on the expression level of the differentially expressed nucleic acid sequence, in another embodiment the system allows the testing of substances directly interacting with the receptor or interfering with the binding of the receptor with a natural or an artificial but appropriate ligand. The latter system comprises a receptor of the invention in a way that a substance which should be tested can physically contact said receptor and which direct interaction leads to a measurable read-out indicative for the change of receptor function.

A method according to the invention comprising a cellular system can be, for example, a method in which a MonoMac6 or a THP-1 cell is used wherein said cell is stimulated with phorbol 12-myristate 13-acetate and with a substance selected from a group consisting of LPS and smoke.

The present invention also provides a test system for determining whether a substance is an activator or an inhibitor according to the invention of an ILM receptor function according to the invention, characterized in that the receptor is involved in a chronic inflammatory airway disease and which receptor plays a role in mediating inflammation, comprising at least an ILM receptor or an expression vector capable of expressing an ILM receptor in a cell or a host cell transformed with an expression vector capable of expressing an ILM receptor.

For performing a method for determining whether a substance is an activator or an inhibitor of receptor function of the present invention cells as well as cell-free systems can be used. Test systems for performing the method can be, for example, designed and built up by using elements and methods well known in the art. For example, cell-free systems may include, for example, cellular compartments or vesicles comprising a receptor of the invention. Suitable cellular systems include, for example, a suitable prokaryotic cell or eukaryotic cell, i.e. comprising a respective receptor of the invention. A cell suitable for performing a said method of the invention may be obtained by recombinant techniques, i.e. after transformation or transfection with a vector suitable for expression of the desired receptor of the invention, or may be a cell line or a cell isolated from a natural source expressing the desired receptor of the invention. A test system according to the invention comprising a cellular system can also be, for example, a test system in which a MonoMac6 or a THP-1 cell is used wherein said cell is stimulated with phorbol 12-myristate 13-acetate and with a substance selected from a group consisting of LPS and smoke. A test system according to the invention may include a natural or artificial ligand of the receptor if desirable or necessary for testing whether a substance of interest is an inhibitor or activator of a receptor of the invention. Test systems of the invention may be available as kits.

A test method according to the invention comprises measuring a read-out, i.e. a phenotypic change in the test system, for example, if a cellular system is used a phenotypic change of the cell. Such change may be a change in a naturally occurring or artificial response of the cell to receptor activation or inhibition, e.g. as detailed in the Examples hereinbelow.

A test method according to the invention can on the one hand be useful for high throughput testing suitable for determining whether a substance is an inhibitor or activator of the invention, but also e.g. for secondary testing or validation of a hit or lead substance identified in high throughput testing.

The present invention also concerns a substance identified in a method according to the invention to be an inhibitor or activator of a receptor of the invention. A substance of the present invention is any compound which is capable of activating or preferably inhibiting a function of a receptor according to the invention. An example of a way to activate or inhibit a function of a receptor is by influencing the expression level of said receptor. Another example of a way to activate or inhibit a function of a receptor is to apply a substance which directly binds the receptor, thereby activating or blocking functional domains of said receptor, which can be done reversibly or irreversibly, depending on the nature of the substance applied.

Accordingly, a substance useful for activating or inhibiting receptor function includes substances acting on the expression of a differentially expressed nucleic acid sequence, but also acting on the receptor itself. Therefore, according to the invention the meaning of the term a "substance of the invention" includes but is not limited to nucleic acid sequences coding for the gene of a receptor of the invention or a fragment or variant thereof and being capable of influencing the gene expression level, e.g. nucleic acid molecules suitable as antisense nucleic acid, ribozyme, or for triple helix formation. Another substance of the invention is e.g. an antibody or an organic or inorganic compound directly binding to or interfering with the binding of an appropriate ligand with a receptor of the invention and thereby affecting its function.

In a further aspect, the present invention relates to a method for determining an expression level of an ILM receptor differentially expressed nucleic acid sequence or protein according to the invention comprising determining the level of said ILM receptor in a macrophage according to the invention. Such a method can be used, for example, for testing whether a substance is capable of influencing differentially expressed nucleic acid sequence expression levels in a method outlined above for determining whether a substance is an activator or inhibitor. A method for determining an expression level of an ILM receptor differentially expressed nucleic acid sequence or protein can, however, also be used for testing the activation status of a macrophage, e.g. for diagnostic purposes or for investigation of the success of treatment of a disease which is caused by the hyperactivated macrophage.

Accordingly, the invention also relates to a method for diagnosis of a chronic inflammatory disease or monitoring of such disease, e.g. monitoring success in treating beings in need of treatment of such disease, comprising determining the level of the receptor expressed in a macrophage according to the invention. Said macrophage is preferably a mammalian, more preferably a human cell. Accordingly, macrophages of the present invention are preferably obtainable from the site of inflammation in a mammal and more preferably from a site of inflammation in a human being.

A method for determining expression levels of a receptor according to the invention can, depending on the purpose of determining the expression level, be performed by known procedures such as measuring the concentration of respective RNA transcripts via hybridization techniques or via reporter gene driven assays such as luciferase assays or by measuring the protein concentration of said receptor using respective antibodies to verify the identity of said protein.

The present invention relates to the use of a substance according to the invention for the treatment of a chronic inflammatory airways disease according to the invention. Another embodiment of the present invention relates to a pharmaceutical composition comprising at least one of the substances according to the invention determined to be an activator or an inhibitor using the method for determining whether the substance is an activator or an inhibitor according to the invention characterized in that the respective receptor according to the invention is overexpressed in a macrophage according to the invention involved in a chronic inflammatory airway disease according to the invention. The composition may be manufactured in a manner that is itself known, e.g. by means of conventional mixing, dissolving, granulating, dragee-making, levigating, powdering, emulsifying, encapsulating, entrapping or lyophilizing processes.

In order to use substances activating or inhibiting according to the invention as drugs for treatment of chronic inflammatory airway diseases, the substances can be tested in animal models for example an animal suffering from an inflammatory airway disorder or a transgenic animal expressing a receptor according to the invention.

Toxicity and therapeutic efficacy of a substance according to the invention can be determined by standard pharmaceutical procedures, which include conducting cell culture and animal experiments to determine the  $IC_{50}$ ,  $LD_{50}$  and  $ED_{50}$ . The data obtained are used for determining the animal or more preferred the human dose range, which will also depend on the dosage



form (tablets, capsules, aerosol sprays ampules, etc.) and the administration route (for example transdermal, oral, buccal, nasal, enteral, parenteral, inhalative, intratracheal, or rectal).

A pharmaceutical composition containing a least one substance according to the invention as an active ingredient can be formulated in conventional manner. Methods for making such formulations can be found in manuals, e.g. "Remington Pharmaceutical Science". Examples for ingredients that are useful for formulating at least one substance according to the present invention are also found in WO 99/18193, which is hereby incorporated by reference.

In a further aspect the invention teaches a method for treating a chronic inflammatory airway disease according to the invention which method comprises administering to a being preferably to a human being in need of such treatment a suitable amount of a pharmaceutical composition comprising at least one substance determined to be an activator or inhibitor according to a method for determining whether a substance is an activator or an inhibitor according to the invention of an ILM receptor according to the invention characterized in that the receptor is overexpressed in a macrophage according to the invention and plays a role in mediating inflammation involved in a chronic inflammatory airway disease according to the invention.

In another embodiment the invention relates to a method for selectively modulating ILM receptor concentration in a macrophage, comprising administering a substance determined to be an activator or inhibitor of a receptor according to the invention.

Included herein are exemplified embodiments, which are intended as illustrations of single aspects of the invention. Indeed, various modifications of the invention in addition to those herein will become apparent to those skilled in the art from the foregoing description and drawings. Such modifications are intended to fall within the scope of the present invention.

All publications and patent applications cited herein are incorporated by reference in their entireties.

## **Examples**

### **Example 1: Comparative Expression Profiling and FPLR-1 Cloning**

The following is an illustration of how comparative expression profiling can be performed in order to identify receptors according to the present invention.

### 1.1. Selection of Patients

Three groups of subjects are studied: healthy non-smokers, healthy smokers and patients with COPD.

In order to assess lung function subjects have to perform spirometry. A simple calculation based on age and height is used to characterise the results. COPD subjects are included if their FEV<sub>1</sub> % predicted is less than 70%. Healthy smokers are age and smoking history matched with the COPD subjects but have normal lung function. Healthy non-smokers have normal lung function and have never smoked. The latter group has a methacholine challenge to exclude asthma. This technique requires increasing doses of methacholine to be given to the subject, with spirometry between each dose. When the FEV<sub>1</sub> falls 20% the test is stopped and the PC<sub>20</sub> is calculated. This is the dose of methacholine causing a 20% fall in FEV<sub>1</sub> and we will require a value of greater than 32 as evidence of absence of asthma. All subjects have skin prick tests to common allergens and are required to have negative results. This excludes atopic individuals. The clinical history of the subjects is monitored and examined in order to exclude concomitant disease.

### 1.2. BAL (bronchoalveolar lavage) Procedure

Subjects are sedated with midazolam prior to the BAL. Local anaesthetic spray is used to anaesthetize the back of the throat. A 7mm Olympus bronchoscope is used. The lavaged area is the right middle lobe. 250 ml of sterile saline is instilled and immediately aspirated. The resulting aspirate contains macrophages.

### 1.3. BAL Processing

BAL is filtered through sterile gauze to remove debris. The cells are washed twice in HBSS, resuspended in 1ml HBSS (Hank's Balanced Salt Solution) and counted. The macrophages are spun to a pellet using 15 ml Falcon blue-cap polypropylene, resuspended in Trizol reagent (Gibco BRL Life Technologies) at a concentration of 1 ml Trizol reagent per 10 million cells and then frozen at -70°C.

### 1.4. Differential Gene Expression Analysis

Total RNA is extracted from macrophage samples obtained according to Example 1.3. Cell suspensions in Trizol are homogenized through pipetting and incubated at room temperature

for 5 minutes. 200µ chloroform per ml Trizol is added, the mixture carefully mixed for 15 seconds and incubated for 3 more minutes at room temperature. The samples are spun at 10,000g for 15 minutes at 4°C. The upper phase is transferred into a new reaction tube and the RNA is precipitated by adding 0.5 ml isopropanol per ml Trizol for 10 minutes at room temperature. Then, the precipitate is pelleted by using a microcentrifuge for 10 minutes at 4°C with 10,000g, the pellet is washed twice with 75% ethanol, air dried and resuspended in DEPC-H<sub>2</sub>O.

An RNA cleanup with Qiagen RNeasy Total RNA isolation kit (Qiagen) is performed in order to improve the purity of the RNA. The purity of the RNA is determined by agarose gelelectrophoresis and the concentration is measured by UV absorption at 260 nm.

5 µg of each RNA is used for cDNA synthesis. First and second strand synthesis are performed with the SuperScript Choice system (Gibco BRL Life Technologies). In a total volume of 11 µl RNA and 1 µl of 100 M T7-(dt)<sub>24</sub> primer, sequence shown in SEQ ID NO:13, are heated up to 70°C for 10 minutes and then cooled down on ice for 2 minutes. First strand buffer to a final concentration of 1X, DTT to a concentration of 10 mM and a dNTP mix to a final concentration of 0.5 mM are added to a total volume of 18 µl. The reaction mix is incubated at 42°C for 2 minutes and 2 µl of Superscript II reverse transcriptase (200 U/µl) are added. For second strand synthesis 130 µl of a mix containing 1.15X second strand buffer, 230 µM dNTPs, 10 U E.coli DNA ligase (10U/µl), E.coli DNA polymerase (10 U/µl), RNase H (2U/l) is added to the reaction of the first strand synthesis and carefully mixed with a pipette. Second strand synthesis is performed at 16°C for 2 hours, then 2 µl of T4 DNA polymerase (5 U/µl) are added, incubated for 5 minutes at 16°C and the reaction is stopped by adding 10 µl 0.5 M EDTA.

Prior to cRNA synthesis the double stranded cDNA is purified. The cDNA is mixed with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) and spun through the gel matrix of phase lock gels (Eppendorf) in a microcentrifuge in order to separate the cDNA from unbound nucleotides. The aqueous phase is precipitated with ammoniumacetate and ethanol. Subsequently, the cDNA is used for *in vitro* transcription. cRNA synthesis is performed with the ENZO BioArray High Yield RNA Transcript Labeling Kit according to manufacturer's protocol (ENZO Diagnostics). Briefly, the cDNA is incubated with 1X HY reaction buffer, 1X biotin labeled ribonucleotides, 1X DTT, 1X RNase Inhibitor Mix and 1X T7 RNA Polymerase in a total volume of 40 µl for 5 hours at 37°C. Then, the reaction mix is purified via RNeasy columns (Qiagen), the cRNA precipitated with ammonium acetate and ethanol and finally resuspended in DEPC-treated water. The concentration is determined via UV spectrometry at 260 nm. The remaining cRNA is incubated with 1X fragmentation buffer (5X

fragmentation buffer: 200 mM Tris acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc) at 94°C for 35 minutes.

For hybridization of the DNA chip 15 µg of cRNA is used, mixed with 50 pM biotin-labeled control B2 oligonucleotide, sequence shown SEQ ID NO:14, 1X cRNA cocktail, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, 1X MES (2-[N-morpholino]-ethanesulfonic acid) hybridization buffer in a total volume of 300 µl. The hybridization mixture is heated up to 99°C for 5 minutes, cooled down to 45°C for 10 minutes and 200 µl of the mix are used to fill the probe array. The hybridization is performed at 45°C at 60 rpm for 16 hours.

After the hybridization the hybridization mix on the chip is replaced by 300 µl non-stringent wash buffer (100 mM MES, 100 mM NaCl, 0.01% Tween 20). The chip is inserted into an Affymetrix Fluidics station and washing and staining is performed according to the EukGE-WS2 protocol. The staining solution per chip consists of 600 µl 1X stain buffer (100 mM MES, 1 M NaCl, 0.05% Tween 20), 2 mg/ml BSA, 10 µg/ml SAPE (streptavidin phycoerythrin) (Dianova), the antibody solution consists of 1X stain buffer, 2 mg/ml BSA, 0.1 mg/ml goat IgG, 3 µg/ml biotinylated antibody.

After the washing and staining procedure the chips are scanned on the HP Gene Array Scanner (Hewlett Packard).

Data Analysis is performed by pairwise comparisons between chips hybridized with RNA isolated from COPD smokers and chips hybridized with RNA isolated from healthy smokers. One of the different expressed nucleic acid sequences identified is coding for FPRL-1 (formyl peptide receptor like-1) receptor (also named LXA<sub>4</sub>R, HM63, FPR2, FPRH2, FMLP-R-II, Lipoxin A<sub>4</sub> receptor); see SEQ ID NOs:1 and 2. It belongs to the chemoattractant peptide receptor family including receptors for fMLP (N-formyl-methionyl-leucyl-phenylalanine), IL-8 or C5a. These receptors show a seven-transmembrane helix motif and signal through heterotrimeric G-proteins. FPRL-1 receptor was identified as the high-affinity receptor for lipoxin A<sub>4</sub> (LXA<sub>4</sub>) (Murphy, P. M. et al. 1992, J. Biol. Chem. 267:7637-7643).

Alveolar macrophages have been shown to produce lipoxins, which are synthesized by 15-lipoxygenase (Kim, S.J., 1988, Biochem. Biophys Res. Commun. 150:870-876). Lipoxin A<sub>4</sub> (LXA<sub>4</sub>) stimulates chemotaxis, adherence and calcium release in monocytes. In neutrophils, though, LXA<sub>4</sub> inhibits chemotaxis and adhesion, and downregulates transmigration through epithelial cells (Maddox, J.F. and Serhan, C.N. 1996, J. Exp. Med. 183:137-146). LXA<sub>4</sub> was found elevated in BALs from patients with asthma (Lee, T.H. et al. 1990, Am. Rev. Respir. Dis. 141:1453-1458 and Serhan, J. N. 1999, Lipoxigenases and Their Metabolites, ed. Nigam and Pace-Asciak, Plenum Press, New York 133-149). In particular, it was found to cause a dose-dependent contraction of human bronchi (Christie et al. 1992, Am. Rev. Respir. Dis. 145:1281-1284). LXA<sub>4</sub> is considered to be a generic modulator of inflammation in the lung.

**1.5. FPRL-1 receptor Overexpressed in COPD Macrophages**

FPRL-1 receptor is consistently found upregulated (66.7%) in COPD smokers compared to healthy smokers. This is demonstrated by calculated “fold change” values from 42 pairwise comparisons and by average difference (“avg diff”) values (Table 1, 2). Relative expression levels for non-smokers and healthy smokers are similar and elevated levels are restricted to patients with COPD. Therefore, COPD-specific effects cause the upregulation.

Table 1: Expression pattern for FPRL-1 receptor: fold change calculation for 42 pairwise comparisons between COPD and healthy smokers. Only values higher than 2fold and lower than -2fold are considered as deregulated. Thus FPRL-1 receptor was 28 times upregulated and 14 times not regulated.

fold change	comparison	fold change	comparison	fold change	comparison
2.7	39vs2	2.9	5vs2	3.3	1vs2
4.6	39vs37	3.6	5vs37	5.5	1vs37
2	39vs43	1.4	5vs43	1.4	1vs43
3.1	39vs56	3	5vs56	3.9	1vs56
4.1	39vs57	3.2	5vs57	5.3	1vs57
2.9	39vs58	3	5vs58	3.6	1vs58
2.2	39vs62	2.7	5vs62	2.7	1vs62
1.3	44vs2	2.7	6vs2	1.4	3vs2
2.7	44vs37	4.1	6vs37	2.9	3vs37
-1.9	44vs43	1.1	6vs43	-1.7	3vs43
1.5	44vs56	3.2	6vs56	1.7	3vs56
2	44vs57	3.5	6vs57	2.3	3vs57
1.4	44vs58	2.9	6vs58	1.5	3vs58
1.1	44vs62	2.2	6vs62	1.2	3vs62

Table 2: Expression levels of FPRL-1 receptor: "avg diff" values, a relative indicator of the intensity of the hybridisation signal on the chip, for each patient are listed; OS means obstructed smoker, HS healthy smoker, NS non-smoker

OS	avg diff	HS	avg diff	NS	avg diff
<u>P 1</u>	<u>1276.7</u>	<u>P 2</u>	<u>490.4</u>	<u>P 48/49</u>	<u>248.2</u>
<u>P 3</u>	<u>553.6</u>	<u>P 37</u>	<u>52.1</u>	<u>P 50/52</u>	<u>565.7</u>
<u>P 5</u>	<u>1710.2</u>	<u>P 43</u>	<u>940</u>	<u>P 54/61</u>	<u>142.4</u>
<u>P 6</u>	<u>1046.9</u>	<u>P 56</u>	<u>327.1</u>		
<u>P 39</u>	<u>1025.2</u>	<u>P 57</u>	<u>238.7</u>		
<u>P 44</u>	<u>507.1</u>	<u>P 58</u>	<u>358.2</u>		
		<u>P62</u>	<u>469.6</u>		

<b>mean + std. dev.</b>		1020.0 ± 452.5		410.9 ± 276.3		318.8 ± 220.3
<b>Median</b>		1036.1		327.1		248.2

P value for comparisons between COPD smokers and healthy smokers: 0.02

Chip data for FPRL-1 receptor are confirmed by TaqMan analysis (Perkin Elmer Applied Biosystems) for three COPD and two healthy smokers. Fold changes obtained by TaqMan very much resemble the data from the gene chips (Table 3).

Table 3: Upregulation of FPRL-1 receptor in COPD smokers determined by gene chips and TaqMan.

Fold change determination for FPRL-1 receptor by chip data in six comparisons between COPD smokers and healthy smokers is validated by analysis of the same samples by TaqMan and the relative upregulation is calculated with GAPDH as a housekeeping gene.

comparison	chip	TaqMan
1vs2	3.3	4.1
3vs2	1.4	2.2
39vs2	2.7	6.0

comparison	chip	TaqMan
1vs37	5.5	4.6
3vs37	2.9	2.5
39vs37	4.6	6.8

Another differentially expressed nucleic acid sequence identified codes for HM74 receptor, see SEQ ID NOs:20 and 21, which belongs to the family of G-protein-coupled receptors. HM74 receptor was cloned from a human monocytic library (Nomura, H. et al. 1993, Internat. Immunol. 5:1239-1249). To date, the ligand has not been identified. HM74 receptor is consistently found upregulated (54.8%) in COPD smokers compared to healthy smokers. This is demonstrated by calculated "fold change" values (Table 5) from 42 pairwise comparisons and by "avg diff" values (Table 6).

Table 5. Expression pattern for HM74 receptor: fold change calculation for 42 pairwise comparisons between COPD and healthy smokers. Only values higher than 2fold and lower than -2fold are considered as deregulated. Thus, HM74 receptor was 23 times upregulated and 17 times not regulated

fold change	comparison	fold change	comparison	fold change	comparison
1.2	39vs2	4.5	5vs2	-1.2	1vs2
4.7	39vs37	13.8	5vs37	2.8	1vs37
-2.1	39vs43	2.5	5vs43	-2.2	1vs43
2.9	39vs56	8.6	5vs56	1.8	1vs56
2.6	39vs57	8.9	5vs57	1.6	1vs57
2.6	39vs58	7.7	5vs58	1.6	1vs58
2.4	39vs62	8.5	5vs62	1.5	1vs62
2.8	44vs2	1	6vs2	-1.1	3vs2
8.8	44vs37	3.5	6vs37	3	3vs37
1.5	44vs43	-1.7	6vs43	-2	3vs43
5.5	44vs56	2.2	6vs56	1.9	3vs56
5.4	44vs57	2	6vs57	1.7	3vs57
4.9	44vs58	1.9	6vs58	1.7	3vs58
5.2	44vs62	1.9	6vs62	1.7	3vs62

Table 6: Expression levels of HM74 receptor: "avg diff" values, a relative indicator of the intensity of the hybridisation signal on the chip, for each patient are listed; OS means obstructed smoker, HS healthy smoker, NS non-smoker

OS	avg diff	HS	avg diff	NS	avg diff
<u>P 1</u>	<u>3233</u>	<u>P 2</u>	<u>3916.3</u>	<u>P 48/49</u>	<u>1690.7</u>
<u>P 3</u>	<u>3474.5</u>	<u>P 37</u>	<u>1154.5</u>	<u>P 50/52</u>	<u>4176.4</u>



<b>P 5</b>	<u>17671</u>	<b>P 43</b>	<u>5770.5</u>	<b>P 54/61</b>	<u>3504.8</u>
<b>P 6</b>	<u>4094.2</u>	<b>P 56</b>	<u>1860.2</u>		
<b>P 39</b>	<u>4201.3</u>	<b>P 57</b>	<u>1639.8</u>		
<b>P 44</b>	<u>11068.5</u>	<b>P 58</b>	<u>2080.2</u>		
		<b>P62</b>	1721.6		

<b>mean + std. dev.</b>		7290.4 ± 5879.0		2591.9 ± 1652.5		3124.0 ± 1285.9
<b>median</b>		4147.8		2243.6		3504.8

Chip data for HM74 receptor are confirmed by TaqMan analysis for three COPD and two healthy smokers. Fold changes obtained by TaqMan very much resemble the data from the gene chips (Table 7).

Table 7: Upregulation of HM74 receptor in COPD smokers determined by gene chips and TaqMan.

Fold change determination for HM74 receptor by chip data in six comparisons between COPD smokers and healthy smokers is validated by analysis of the same samples by TaqMan and the relative upregulation is calculated with GAPDH as a housekeeping gene.

<b>comparison</b>	<b>chip</b>	<b>TaqMan</b>
1vs2	0.8	2.3
3vs2	0.9	0.8
39vs2	1.2	1.4

<b>comparison</b>	<b>chip</b>	<b>TaqMan</b>
1vs37	2.8	4.5
3vs37	3.0	1.4
39vs37	4.7	2.6

Another differentially expressed nucleic acid sequence identified codes for AICL receptor (activation-induced C-type lectin), see SEQ ID NOs:5 and 6., which is a type II membrane protein that recognizes and binds N-acetyl-galactosamin or -glucosamin moieties of plasma glycoproteins (Oda, S. et al. 1988, J. Biochem. 104:600-605). It is expressed in lymphoid tissues and in hematopoietic cells as well as in NK and T cells. Its expression is induced during lymphocyte activation and after stimulation with PMA (Hamann, J. et al. 1997, Immunogenetics 45:295-300). Since homologues of AICL receptor are involved in signal transmission in lymphocytes and in lymphocyte proliferation, it is tempting to assume that

AICL receptor also participates in these processes (Hamann, J. et al. 1993, Immunol. 150:4920:4927).

AICL receptor is consistently found upregulated (66.7%) in COPD smokers compared to healthy smokers. This is demonstrated by calculated “fold change” values (Table 8) from 42 pairwise comparisons and by “avg diff” values (Table 9). The p value for the comparisons between COPD smokers and healthy smokers was 0.01.

**Table 8.** Expression pattern for AICL receptor: fold change calculation for 42 pairwise comparisons between COPD and healthy smokers. Only values higher than 2fold and lower than -2fold are considered as deregulated. Thus, AICL receptor was 28 times upregulated and 14 times not regulated

fold change	comparison	fold change	comparison	fold change	comparison
1	39vs2	1.5	5vs2	-1.3	1vs2
1.9	39vs37	2.8	5vs37	1.4	1vs37
-1.4	39vs43	2.4	5vs43	1.3	1vs43
3.3	39vs56	5	5vs56	2.7	1vs56
6.9	39vs57	10	5vs57	5.3	1vs57
3.1	39vs58	4.5	5vs58	2.3	1vs58
3.3	39vs62	5.1	5vs62	2.7	1vs62
1.4	44vs2	-1.4	6vs2	-1.5	3vs2
2.6	44vs37	1.2	6vs37	1.2	3vs37
2.3	44vs43	1.1	6vs43	1.1	3vs43
4.2	44vs56	2.3	6vs56	2.3	3vs56
9.6	44vs57	4.5	6vs57	4.5	3vs57
4.3	44vs58	2	6vs58	2	3vs58
4.2	44vs62	2.3	6vs62	2.3	3vs62

Table 9: Expression levels of AICL receptor: "avg diff" values, a relative indicator of the intensity of the hybridisation signal on the chip, for each patient are listed; OS means obstructed smoker, HS healthy smoker, NS non-smoker

OS	avg diff	HS	avg diff	NS	avg diff
P 1	3415.3	P 2	4984.2	P 48/49	748.4
P 3	3412.9	P 37	2388.6	P 50/52	1726.5
P 5	6585.8	P 43	2722.5	P 54/61	1087.9
P 6	3444.7	P 56	1121.1		
P 39	4548.4	P 57	656.1		
P 44	6291.5	P 58	1476.0		
		P 62	1113.1		

mean + std. dev.		4622.4 ± 1474.3		2065.9 ± 1482.0		1187.5 ± 496.6
median		3996.6		1476.0		1087.9

Another differentially expressed nucleic acid sequence identified codes for ILT1 receptor (immunoglobulin-like transcript 1), see SEQ ID NOs:11 and 12. ILT1 receptor belongs to the Ig superfamily receptors that is related to a subset of activating receptors similar to NK cell receptors for MHC class I molecules. ILT1 receptor is a 69 kDa glycosylated transmembrane receptor which is mainly expressed in lung and liver and in monocytes, granulocytes, macrophages, and dendritic cells (Samaridis, J. and Colonna, M., 1997, Eur. J. Immunol. 27:660-665). Upon crosslinking with antibodies ILT1 receptor interacts with the  $\gamma$ -chain of the Fc receptor (Fc $\epsilon$ RI $\gamma$  (Nakajima et al., 1999 J. Immunol. 162(1):5-8)

ILT1 receptor is found consistently upregulated (59.5%) in COPD smokers compared to healthy smokers. This is demonstrated by "avg diff" values (Table 10). The p value for the comparisons between COPD smokers and healthy smokers was 0.01.

Table 10: Expression levels of ILT1 receptor: "avg diff" values for each patient are listed as well as mean and median values for the three groups of subjects; OS means obstructed smoker, HS healthy smoker, NS non-smoker

OS	avgdiff	HS	avg diff	NS	avg diff
P 1	493.5	P 2	412.3	P 48/49	519.7
P 3	1186.0	P 37	457.2	P 50/52	645.0
P 5	1097.1	P 43	382.6	P 54/61	491.2
P 6	1387.6	P 56	180.5		

<b>P 39</b>	513.5	<b>P 57</b>	367.8		
<b>P 44</b>	1374.5	<b>P 58</b>	720.8		
		<b>P 62</b>	279.1		

<b>mean + std. dev.</b>		1008.8 ± 406.8		400.0 ± 168.6		552.0 ± 81.8
<b>median</b>		1141.6		382.6		519.7

Another differentially expressed nucleic acid sequence identified codes for SHPS-1 receptor (SIRP- $\alpha$ 1, MYD1, MFR), see SEQ ID NOs:3 and 4, which is known to be highly expressed in macrophages (Fujioka, Y. et al. 1996, Mol. Cell. Biol. 16:6887-6899 and Kharitononkov, A. et al. 1997, Nature 386:181-186; Brooke, G. P. et al. 1998, Eur J. Immunol. 28:1:11). SHPS-1 receptor is a transmembrane glycoprotein belonging to immunoglobulin superfamily. It contains three extracellular Ig-like domains, a cytoplasmic tail with a potential tyrosine phosphorylation site and an immunoreceptor tyrosine-based inhibitory motif (ITIM). Tyrosine phosphorylation of SHPS-1 receptor occurs upon activation of receptor tyrosine kinases and leads to an association with SHP-1 (in macrophages) and SHP-2 (in non-hematopoietic cells) (Veillette, A. et al. 1998, J. Biol. Chem. 273:22719-22728). Moreover, other proteins have been found to associate with the intracytoplasmic domain of SHPS-1 receptor, and it is therefore tempting to assume that SHPS-1 receptor acts as a scaffolding protein.

SHPS-1 receptor is consistently found downregulated (73.8%) in COPD smokers compared to healthy smokers. This is demonstrated by calculated "fold change" values (Table 11) from 42 pairwise comparisons and by "avg diff" values (Table 12). The p value for the comparisons between COPD smokers and healthy smokers is 0.005.

Table 11. Expression pattern for SHPS-1 receptor: fold change calculation for 42 pairwise comparisons between COPD and healthy smokers. Only values higher than 2fold and lower than -2fold are considered as deregulated. Thus, SHPS-1 receptor is 29 times downregulated and 13 times not regulated

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fold change	comparison	fold change	comparison	fold change	comparison
-1.3	39vs2	-3.4	5vs2	1.3	1vs2
-2.8	39vs37	-6.8	5vs37	-1.7	1vs37
-1.6	39vs43	-8.4	5vs43	-2.1	1vs43
-3.0	39vs56	-7.1	5vs56	-1.8	1vs56
-5.6	39vs57	-13.2	5vs57	-3.4	1vs57
-5.4	39vs58	-12.6	5vs58	-3.2	1vs58
-3.1	39vs62	-7.5	5vs62	-1.9	1vs62
1.4	44vs2	-2.1	6vs2	-1.1	3vs2
-1.5	44vs37	-4.5	6vs37	-2.3	3vs37
-1.8	44vs43	-5.6	6vs43	-2.9	3vs43
-1.6	44vs56	-4.7	6vs56	-2.4	3vs56
-2.6	44vs57	-8.9	6vs57	-4.6	3vs57
-2.5	44vs58	-8.5	6vs58	-4.4	3vs58
-1.7	44vs62	-4.9	6vs62	-2.5	3vs62

Table 12: Expression levels of SHPS-1 receptor: "avg diff" values for each patient are listed as well as mean and median values for the three groups of subjects; OS means obstructed smoker, HS healthy smoker, NS non-smoker

OS	avg diff	HS	avg diff	NS	avg diff
P 1	1837.8	P 2	1442.6	P 48/49	4979.9
P 3	1361.1	P 37	3115.0	P 50/52	1120.5
P 5	291.1	P 43	3897.3	P 54/61	2090.6
P 6	696.3	P 56	3280.8		
P 39	1105.4	P 57	6220.7		
P 44	2466.0	P 58	5928.9		
		P 62	3431.7		

mean + std. dev.		1293.0 ± 783.9		3902.4 ± 1671.3		2730.3 ± 2007.7
median		1233.4		3431.7		2090.6

Another differentially expressed nucleic acid sequence identified codes for KDEL receptor 1, see SEQ ID NOs:7 and 8, which is a receptor that has important functions in protein folding and assembly in the endoplasmic reticulum. It recognizes soluble proteins with the amino acid sequence K-D-E-L and retrieves these proteins after binding to the endoplasmic reticulum (Townesley, F. M. et al. 1993, EMBO J. 12:2821-2829). KDEL receptor 1 may be involved in the regulation of protein transport in the Golgi complex. Upon binding of a ligand the KDEL receptor dimerizes and interacts with ARF GAP (GTPase-activating protein for the ADP-ribosylation factor) (Aoe, T. et al 1997, EMBO J. 16:7305-7316).

It is consistently found downregulated (71.4%) in COPD smokers compared to healthy smokers. This is shown by "avg diff" values (Table 13). The p value for the comparisons between COPD smokers and healthy smokers is 0.003.

Table 13: Expression levels of KDEL receptor 1: "avg diff" values for each patient are listed as well as mean and median values for the three groups of subjects; OS means obstructed smoker, HS healthy smoker, NS non-smoker

OS	avg diff	HS	avg diff	NS	avg diff
P 1	877.6	P 2	930.6	P 48/49	1532.9
P 3	1227.2	P 37	2151.4	P 50/52	786.4
P 5	870.6	P 43	1628.6	P 54/61	1571.5

<b>P 6</b>	1188.6	<b>P 56</b>	2232.9		
<b>P 39</b>	1404.5	<b>P 57</b>	2295.1		
<b>P 44</b>	798.1	<b>P 58</b>	2364.1		
		<b>P 62</b>	2092.0		

<b>mean + std. dev.</b>		1061.1 ± 245.3		1956.4 ± 512.1		1296.9 ± 442.6
<b>median</b>		1033.1		2151.4		1532.9

Another differentially expressed nucleic acid sequence identified codes for the macrophage colony-stimulating factor-1 receptor precursor (CSF-1 receptor, c-fms); see SEQ ID NOs:9 and 10. The CSF-1 receptor belongs to the subfamily of receptor tyrosine kinases. Activation of the CSF-1 receptor results in complex formation of multiple proteins, e.g. CSF-1 receptor, Shc, PI3K, Grb2, Cbl, SHP-1, Src. Moreover, ligand binding also triggers rapid tyrosine phosphorylation of a plethora of cytoplasmic proteins like Cbl, STAT3, STAT5a, STAT5b, p85PI3K, SHP-1, Vav and proteins involved in cytoskeletal organization (Yeung, Y.-G. et al. 1998, J. Biol. Chem. 273:17128-17137). CSF-1 receptor regulates survival, proliferation, differentiation and morphology of mononuclear phagocytes (Hampe, A. et al. 1989, Oncogene Res. 4:9-17).

CSF-1 receptor is consistently found downregulated (45.2%) in COPD smokers compared to healthy smokers. This is shown by “avg diff” values (Table 14). The p value for the comparisons between COPD smokers and healthy smokers is 0.002.

Table 14: Expression levels of CSF-1 receptor: “avg diff” values for each patient are listed as well as mean and median values for the three groups of subjects; OS means obstructed smoker, HS healthy smoker, NS non-smoker

<b>OS</b>	<b>avg diff</b>	<b>HS</b>	<b>avg diff</b>	<b>NS</b>	<b>avg diff</b>
<b>P 1</b>	1136.0	<b>P 2</b>	2591.4	<b>P 48/49</b>	2967.7
<b>P 3</b>	2262.5	<b>P 37</b>	3070.6	<b>P 50/52</b>	2041.6
<b>P 5</b>	829.5	<b>P 43</b>	2799.2	<b>P 54/61</b>	2376.4
<b>P 6</b>	1720.3	<b>P 56</b>	3293.1		
<b>P 39</b>	1860.7	<b>P 57</b>	3703.4		
<b>P 44</b>	1334.1	<b>P 58</b>	1904.9		
		<b>P 62</b>	2144.5		

<b>mean + std. dev.</b>		1523.9 ± 522.7		2786.7 ± 633.2		2461.9 ± 468.9
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<b>median</b>		1527.2		2799.2		2376.4
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#### 1.6. Use of TaqMan Analysis for Validation of DNA-Chip Data and Diagnosis

mRNA-expression profiles obtained by DNA-chips are validated by TaqMan analysis with the same RNA preparations. Moreover, the method is also applied to determine mRNA-levels for FPRL-1 receptor in cultured cell lines and in cells isolated from human beings in order to monitor the progress of the disease.

Total RNA isolated from U937-cells that were treated for 3 days with 10 nM retinoic acid is used in order to optimize of reaction conditions for determining the mRNA-levels of FPRL-1 receptor and setting standard curves for FPRL-1 receptor and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a housekeeping gene. Quantification of FPRL-1 receptor is done with the following primers: Forward primer (FP) see SEQ ID NO:17, Reverse primer (RP) see SEQ ID NO:18 and TaqMan probe (TP) see SEQ ID NO:19 labeled with reporter dye FAM at the 5' end and quencher dye TAMRA at the 3' end. For determining mRNA-levels for GAPDH a predeveloped kit "TaqMan GAPDH Control Reagents" (P/N 402869) from Perkin Elmer Applied Biosystems is used. The GAPDH probe is labeled with JOE as the reporter dye and TAMRA as the quencher dye. RT-PCR reactions are performed with the "TaqMan EZ RT-PCR Core Reagents" (P/N N808-0236) kit from Perkin Elmer Applied Biosystems. Standard curves for FPRL-1 receptor and GAPDH are performed with increasing concentrations of RNA from U937 cells treated with 1  $\mu$ M retinoic acid ranging from 0, 5, 10, 25, 50 to 100 ng per assay. Reaction mixes contain 1X TaqMan EZ-buffer, 3 mM Mn(Oac)<sub>2</sub>, 300  $\mu$ M dATP, dCTP, dGTP, and 600  $\mu$ M dUTP, 2.5 U rTth DNA polymerase, 0.25 U AmpErase UNG in a total volume of 25  $\mu$ l. For analysis of FPRL-1 receptor reaction mixes include 300 nM of FP and RP and 100 nM of TP. The primer concentrations for determining GAPDH levels are 200 nM for each primer and 100 nM for the GAPDH Taqman probe. In order to determine mRNA levels for FPRL-1 receptor and GAPDH in human subjects and cell lines 16 to 50 ng RNA per reaction are used. All samples are run in triplicate. The reactions are performed with "MicroAmp Optical 96-well reaction plates" sealed with "MicroAmp Optical Caps" (Perkin Elmer Applied Biosystems) in an ABI PRISM 7700 Sequence Detection System (Perkin Elmer Applied Biosystems). The PCR conditions are 2 minutes at 50°C, 30 minutes at 60°C, 5 minutes at 95°C, followed by 40 cycles of 20 seconds at 94°C and 1 minute at 59°C. Data analysis is done either by determining the mRNA levels for FPRL-1 receptor and GAPDH according to the standard curves or by directly relating C<sub>T</sub> values for FPRL-1 receptor to C<sub>T</sub> values for GAPDH. The latter can be done for these genes since the efficiencies for both reactions are around 95%. The same method is used for investigating mRNA levels isolated from COPD patients in order to diagnose the disease or,



after treatment of patients with their putative active drugs to monitor the success of the treatment.

The other receptors mentioned in example 1.5 are investigated accordingly by using the respective appropriate primers.

### 1.7. Cell Systems

Human monocytic/macrophage cell lines HL-60, U937, THP-1, and MonoMac 6 are used as cellular model systems. Cells are grown in RPMI 1640 media containing 10% FCS supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 1X non-essential amino acids. The media for MonoMac6 cells also includes 5 ml/l OPI media supplement (Sigma). MonoMac6 cells are exclusively cultured in 24-well plates. Cells are maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C and tested regularly for contamination by mycoplasma.

Differentiation is achieved by adding 10 nM PMA (phorbol 12 myristate-13 acetate) to the media.

### 1.8. Cloning of FPRL-1 receptor

FPRL-1 receptor is cloned from a total RNA extracted from U937 cells that were treated with 1 µM retinoic acid for three days. 5 µg RNA is reverse transcribed into cDNA with 5 ng oligo(dt)<sub>18</sub> primer, 1X first strand buffer, 10 mM DTT, 0.5 mM dNTPs and 2 U Superscript II (Gibco BRL Life Technologies) at 42°C for 50 minutes. Then, the reaction is terminated at 70°C for 15 minutes and the cDNA concentration is determined by UV-spectrophotometry. For amplification of FPRL-1 receptor 100 ng of the cDNA and 10 pmol of sequence-specific primers for FPRL-1 receptor (forward primer attB1; see SEQ ID NO:15 and reverse primer attB2 ; see SEQ ID NO:16) are used for PCR. Reaction conditions are: 2 minutes of 94°C, 35 cycles with 30 seconds at 94°C, 30 seconds at 53°C, 90 seconds at 72°C, followed by 7 minutes at 72°C with Taq DNA-polymerase. The reaction mix is separated on a 2% agarose gel, a band of about 1,000 bp is cut out and purified with the QIAEX II extraction kit (Qiagen). The concentration of the purified band is determined and about 120 ng are incubated with 300 ng of pDONR201, the donor vector of the Gateway system (Gibco BRL Life Technologies), 1X BP clonase reaction buffer, BP clonase enzyme mix in a total volume of 20 µl for 60 minutes at 25°C. Then, reactions are incubated with 2 µl of proteinase K and incubated for 10 minutes at 37°C. The reaction mix is then electroporated into competent DB3.1 cells and plated on Kanamycin-containing plates. Clones are verified by sequencing. A clone, designated pDONR-HM63 carrying the nucleic acid sequence shown in SEQ ID NO:1 is used for further experiments.

The other receptors mentioned in example 1.5 are cloned using analogous methods.

### 1.9. Transfection of FPRL-1 receptor

The vector containing FPRL-1 receptor described under 1.8 is used to transfer the cDNA for FPRL-1 receptor to the expression vector pcDNA3.1(+)/attR that contains the "attR1" and "attR2" recombination sites of the Gateway cloning system (Gibco BRL Life Technologies) where FPRL-1 receptor is expressed under the control of the CMV promoter. 150 ng of the "entry vector" pDONR-HM63 is mixed with 150 ng of the "destination vector" pcDNA3.1(+)/attR, 4 µl of the LR Clonase enzyme mix, 4 µl LR Clonase reaction buffer, added up with TE (Tris/EDTA) to 20 µl and incubated at 25°C for 60 minutes. Then, 2 µl of proteinase K solution is added and incubated for 10 minutes at 37°C. 1 µl of the reaction mix is transformed into 50 µl DH5 by a heat-shock of 30 seconds at 42°C after incubating cells with DNA for 30 minutes on ice. After heat-shock of the cells 450 µl of S.O.C. is added and cells are incubated at 37°C for 60 minutes. Cells (100 µl) are plated on LB plates containing 100 µg/ml ampicillin and incubated over night.

A colony that contains pcDNA3.1(+)/attR with FPRL-1 receptor as an insert is designated pcDNA/FPRL1 and used for transfection studies.

Cell clones containing vectors obtained in 1.8 carrying nucleic acid sequences coding for the other receptors described 1.5 are prepared using analogous methods.

### Example 2: Cellular Systems and Phenotypic Effects of FPRL-1 receptor

Analogous methods as described herein in example 2 for FPRL-1 receptor are also performed using the other receptors described in 1.5.

#### 2.1. Cell Systems

Human monocytic/macrophage cell lines HL-60, U937, THP-1, and MonoMac6 are used as cellular model systems. Cells are grown in RPMI 1640 media containing 10% FCS supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 1X non-essential amino acids. The media for MonoMac6 also includes 5 ml/l OPI media supplement (Sigma). MonoMac6 cells are exclusively cultured in 24-well plates. All cells are maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C and tested regularly for contamination by mycoplasma.

Differentiation is achieved by adding 10 nM PMA (phorbol 12 myristate-13 acetate) to the media.

#### Phenotypic effects of FPRL-1 receptor (2.2.-2.9.)

##### 2.2. Ligand Binding Assay

300 ml cell culture is harvested with EDTA solution, the suspension is used to spin down the cells at 110-220 X g, resuspended in 10 mM Tris/HCl, pH 7.4, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 40 µg/ml bacitracin, 4 µg/ml leupeptin, 4 µg/ml chymostatin, 10 µg/ml pefabloc, 2 µM phosphoramidon and 0.1 mg/ml bovine serum albumin (BSA Fraktion V, BI Bioproducts) and diluted to  $2 \times 10^6$  cells/ml.

0.5 ml aliquots are incubated with 0.3 nM <sup>3</sup>H-lipoxinA4 (specific activity approximately 10 Ci/mmol) or in the presence of increasing concentrations of untritiated lipoxin A4 (3-300 nM) for 30 minutes at 4°C. The incubation is terminated by harvesting the cells by a Cell-Harvester (Skatron) with GF/B filters, washed three times with 3 ml chilled buffer consisting of 50 mM Tris/HCl, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, pH 7.4 and the filter-pieces transferred in vials. 2 ml scintillation cocktail is added and the radioactivity determined with a scintillation counter (LKB). Non-specific binding is determined in the presence of 100 nM unlabeled lipoxinA4. A series of peptides and low molecular weight compounds, including the peptide ligand MMK-1 (Klein, C. et al. 1998, Nature Biotech. 16:1334-1337), is used in a concentration range of 0.5 to 300 nM under the same reaction conditions in order to displace tritiated lipoxin A4.

The bound radioactivity (on the filter pieces) is estimated with a counter, the values are recorded on-line and fitted to a model. IC<sub>50</sub> values for any substance to block binding of <sup>3</sup>H-lipoxin A4 are calculated.

##### 2.3. Ca<sup>2+</sup>-Release Determined by FLIPR-Assay

FLIPR-assay (Fluorometric Imaging Plate Reader) with FPRL-1 receptor is performed with different CHO cell lines that constitutively express the G-protein  $\alpha$ -subunit  $\alpha 16$  or the chimeric G-proteins Gqi5 or Gqo5 (these are two G $\alpha$ (q) chimeras harboring the last five residues of G $\alpha$ (i) or G $\alpha$ (o)) and FPRL-1 receptor. The cell lines CHO/Galpha16 (CHO/Galpha16), CHO/GalphaGqi5 and CHO/GalphaGqo5 (Boehringer Ingelheim) that constitutively express G $\alpha 16$ , Gqi5 or Gqo5 are transfected with the FPRL-1 receptor expression vector. The cell lines are cultured in Ham's F12 media (Bio Whittaker) with 10% FCS (fetal calf serum), 2 mM glutamine, 200 ng/ml hygromycin, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C.  $3-7 \times 10^5$  cells are

seeded in a 60 mm petri dish and grown over night. Cells that are grown to a confluency of 50-80% are used for transfection. 6  $\mu$ l FuGene6 (Roche Biochemicals) is added to 100  $\mu$ l of culture media without serum and equilibrated for 5 minutes at room temperature. Then, 2  $\mu$ g of purified pcDNA/FPRL-1 receptor is added to the prediluted FuGene6 solution, gently mixed, and further incubated at room temperature for 15 minutes. The media is aspirated from the cells and 4 ml of fresh media is added to the cells. The FuGene6/DNA solution is added dropwise to the cells and distributed evenly by swirling of the media. After 48 hours the media is aspirated and replaced by Ham's F12 media, 10% FCS, 2 mM glutamine, 200 ng/ml hygromycin, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 200  $\mu$ g/ml G418. During the following five days the media is replaced daily until dead cells and debris is washed out until single colonies of cells are visible. Single colonies are isolated by separation with cloning cylinders and releasing them from the surface by addition of 100  $\mu$ l of 1X trypsin/EDTA. Cells are transferred from the cloning cylinders to 4 ml of media and plated in 6 well-plates. Single clones are expanded and the expression of FPRL-1 receptor in several clones is tested via ligand binding assay (2.2.). The cell clone denoted CHO/Galpha16/FPRL-1 receptor, CHO/GalphaGqi5/FPRL-1 receptor, or CHO/GalphaGqo5/FPRL-1 receptor with the highest expression of FPRL-1 receptor is used for measuring of intracytoplasmic  $\text{Ca}^{2+}$  via FLIPR (Molecular Devices).

Cells (CHO/Galpha16/FPRL-1 receptor, CHO/GalphaGqi5/FPRL-1 receptor, or CHO/GalphaGqo5/FPRL-1 receptor) are seeded in 384-blackwell plates (Corning) with 2500-5,000 cells per well in a volume of 40  $\mu$ l and grown overnight in a humidified atmosphere with 5%  $\text{CO}_2$  at 37°C. As a negative control CHO/Galpha16, CHO/GalphaGqi5 or CHO/GalphaGqo5 cells are used. Then, 40  $\mu$ l of a Fluo-4 (Molecular Probes) staining solution is added to each well in order to label the cells with Fluo-4 at a final concentration of 2  $\mu$ M. The Fluo-4 staining solution is composed of 10.5 ml cell culture media described above, 420  $\mu$ l Probenicid solution (1.42 g Probenicid (Sigma), 10 ml 1 M NaOH, 10 ml Hanks buffer), 42  $\mu$ l Fluo-4 stock solution (50  $\mu$ g Fluo-4, 23 l DMSO, 23  $\mu$ l Pluronic F-127 (20% in DMSO) (Molecular Probes), and 420  $\mu$ l 1M HEPES. After 45 minutes incubation in a humidified atmosphere with 5%  $\text{CO}_2$  at 37°C wells are washed with a EMBLA-washer (4 wash steps, program 03) using 2,000 ml Hanks buffer containing 20 ml Probenicid solution as a wash solution and leaving 25  $\mu$ l wash buffer in each well. Then FLIPR is set to 10,000 counts for stained wells and a difference of 1:5 between unstained and stained wells. Then, 25  $\mu$ l lipoxin A4 and a series of ligands, peptides, and low molecular weight compounds, including the peptide ligand MMK-1 is added to the wells in increasing concentrations (0.5 - 300 nM) diluted in Hanks' buffer/0.1% BSA. Substances according to the invention are tested in increasing concentrations (0.5 - 300 nM) to compete with lipoxin A4 (50 nM) in order to

determine their antagonistic potential. Fluorescence is recorded starting with the addition of the ligand every second for 60 seconds and every 5 seconds for a further 60 seconds.

#### 2.4. Production and Release of Cytokines or Matrix Metalloproteases

Cells of monocytic/macrophage cell lines are treated with lipoxin A4 at cell densities between  $2.5$  and  $5 \times 10^5$  cells/ml. Cells are harvested after 0, 1, 3, 6, 12, 24, 48, and 72 hours, the supernatant frozen for further investigation, cells are washed with PBS, and resuspended in 400  $\mu$ l of RLT buffer (from Qiagen RNeasy Total RNA Isolation Kit) with 143 mM  $\beta$ -mercaptoethanol, the DNA sheared with a 20 g needle for at least 5 times and stored at  $70^\circ\text{C}$ . Total RNAs are isolated with the Qiagen RNeasy Total RNA Isolation Kit (Qiagen) according to the manufacturer's protocol. Purified RNA is used for TaqMan analysis. The expression levels of cytokines  $\text{TNF}\alpha$ , IL- $1\beta$ , IL-8, IL-6, and human matrix metalloproteases, MMP-1, MMP-7, MMP-9, MMP-12, are measured using appropriate primer sequences.

##### 2.4.1. Detection of Secreted Cytokines

Proteins in the supernatants of the cultured and stimulated cells are precipitated by adding TCA (trichloroacetic acid) to a final concentration of 10%. Precipitates are washed twice with 80% ethanol and pellets are resuspended in 50 mM Tris/HCl, pH 7.4, 10 mM  $\text{MgCl}_2$ , 1 mM EDTA. Protein concentration is determined via the Bradford method and 50  $\mu$ g of each sample are loaded on 12% SDS polyacrylamide gels. Gels are blotted onto PVDF-membranes, blocked for 1 hour in 5% BSA in TBST, and incubated for 1 hour with commercially available antibodies against human  $\text{TNF}\alpha$  (tumor necrosis factor  $\alpha$ ) IL- $1\beta$  (interleukin- $1\beta$ ), IL-8 (interleukin 8), and IL-6 (interleukin 6). After washing with TBST blots are incubated with anti-human IgG conjugated to horseradish-peroxidase, washed again and developed with ECL chemiluminescence kit (Amersham). Intensity of the bands are visualised with BioMax X-ray films (Kodak) and quantified by densitometry.

##### 2.4.2. Detection and Activity of Secreted Matrix Metalloproteases

The procedure is identical to the one described in 2.4.1. Antibodies used for Western blotting are against human MMP-1, MMP-7, MMP-9, and MMP-12.

Protease activity is determined with a fluorescent substrate. Supernatants isolated from stimulated and unstimulated cells (described above) are incubated in a total volume of 50  $\mu$ l with 1  $\mu$ M of the substrate (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Glu (EDANS)-Ala-Lys-NH<sub>2</sub> (Novabiochem)) for 5 minutes at room temperature. Positive controls are performed with 125

ng purified MMP-12 per reaction. Protease activity is determined by fluorometry with an excitation at 320 nm and an emission at 405 nm.

In an alternative assay to determine proteolytic activity and cell migration a chemotaxis chamber is used. In the wells of the upper part of the chamber cells ( $10^5$  cells per well) are plated on filters coated with an 8  $\mu$ m layer of Matrigel (Becton Dickinson). In the lower compartment chemoattractants like lipoxin A4 (100 nM), MCP-1 (monocyte chemotactic protein 1) (10 ng/ml) are added to the media. After five days filters are removed, cells on the undersurface that have traversed the Matrigel are fixed with methanol, stained with the Diff-Quik staining kit (Dade Behring) and counted in three high power fields (400X) by light microscopy.

### 2.5. Chemotaxis Assay

In order to determine chemotaxis a 48 well chemotaxis (Boyden) chamber (Neuroprobe) is used. Cells are starved for 24 hours in RPMI media without FCS. Chemoattractants, (50 ng/ml IL-8, 10 ng/ml MCP-1, 10 nM lipoxin A4, 10 nM MMK-1 peptide (2.3.)) are diluted in RPMI media without FCS and 30  $\mu$ l is placed in the wells of the lower compartment. The upper compartment is separated from the lower compartment by a polycarbonate filter (pore size 8  $\mu$ m). 50  $\mu$ l cell suspension ( $5 \times 10^4$ ) are placed in the well of the upper compartment. The chamber is incubated for 5 hours at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Then the filter is removed, cells on the upper side are scraped off, cells on the downside are fixed for 5 minutes in methanol and stained with the Diff-Quik staining set (Dade Behring). Migrated cells are counted in three high-power fields (400X) by light microscopy.

### 2.6. Adherence Assay

Cells are harvested, washed in PBS and resuspended ( $4 \times 10^6$ /ml) in PBS and 1  $\mu$ M BCECF ((2-7-bis-(carboxethyl)-5(6)-carboxyfluorescein acetoxymethyl) ester, Calbiochem) and incubated for 20 minutes at 37°C. Cells are washed in PBS and resuspended ( $3.3 \times 10^6$ /ml) in PBS containing 0.1% BSA.  $3 \times 10^5$  cells (90  $\mu$ l) are added to each well of a 96-well flat bottom plate coated with laminin (Becton Dickinson) and allowed to settle for 10 minutes. 10  $\mu$ l of agonist (100 nM lipoxin A4 plus lipoxin A4 antagonist) are added and plates are incubated for 20 minutes at 37°C. Then, cells are washed with PBS containing 0.1% BSA and adherent cells are solubilized with 100  $\mu$ l of 0.025 M NaOH and 0.1% SDS. Quantification is performed by fluorescence measurement.

## 2.7. Phagocytosis

Cell suspensions ( $2.5 \times 10^4$  cells/ml) are seeded in 6-well plates with 5 ml of U937 or THP-1 or in 24-well plates with 2 ml of MonoMac6 and incubated for 1 hour at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in the presence of agonists (100 nM lipoxin A4, 50 nM MMK-1 peptide (2.3.)) and low molecular weight compounds according to the invention in order to antagonize agonistic effects. 40 µl of a dispersed suspension of heat-inactivated *Saccharomyces boulardii* (20 yeast/cell) are added to each well. Cells are incubated for three more hours, washed twice with PBS and cytocentrifuged. The cytospin preparations are stained with May-Grünwald-Giemsa and phagocytosed particles are counted by light microscopy.

### Example 3: Cell Culture Model for Macrophages isolated from COPD patients

Analogous methods as described here in Example 3 for FPRL-1 receptor are also performed using receptors described in 1.5.

As a cell culture model for macrophages isolated from COPD patients we select the monocytic cell lines MonoMac6 and THP-1. In order to mimic a hyperactivated status of these cell lines, cells are treated with PMA. Cells are exposed to further stimuli that are to mimic a condition that is similar to the situation in COPD. These stimuli are exposure to smoke or to LPS.

#### **Expression of FPRL-1 after stimulation of MonoMac6 cells with PMA, smoke, and LPS**

MonoMac6 cells are cultivated in 24-well plates in RPMI 1640 media, supplemented with 10% FCS (low endotoxin), 2 mM glutamine, 1X non-essential amino acids, 200 U/ml penicillin, 200 µg/ml streptomycin, and 5 ml OPI media supplement (Sigma). Cells are grown to a density of 600,000 cells per well (2 ml media) and stimulated with 10 nM PMA (phorbol 12-myristate 13-acetate) (Sigma), or 20 ng/ml LPS (lipopolysaccharides from *Salmonella minnesota* Re595) (Sigma). For smoke exposure, cells are incubated in media enriched with smoke for 10 minutes at 37°C, 5% CO<sub>2</sub> at a density of  $1 \times 10^6$  cells/ml.

Enrichment of RPMI 1640 media with smoke is performed with the smoke of two cigarettes. The smoke of the cigarettes is pulled into a 50 ml syringe (about 20 volumes of a 50-ml

syringe per cigarette) and then perfused into 100 ml of RPMI 1640 media without supplements. Afterwards, the pH of the smoke-enriched media is adjusted to 7.4 and the media is sterilized through a 0.2  $\mu$ m filter before use. After the exposure with smoke cells are washed at least twice with RPMI 1640 in order to remove residual smoke particles. Then cells are seeded in 24-well plates with 400,000-600,000 cells per well filled with 2 ml of fresh RPMI 1640 media including the supplements mentioned above.

THP-1 cells are grown in 75 cm<sup>2</sup> flasks in RPMI 1640 Glutamax supplemented with 10% FCS (low endotoxin), 200 U/ml penicillin, 200  $\mu$ g/ml streptomycin. Cells are treated with 10 nM PMA for 48 hours at 37°C, 5% CO<sub>2</sub> in order to differentiate the cells to a macrophage-like cell type. Then, media is replaced by new PMA-free cultivation media with the addition of 20 ng/ml LPS.

Both cell types are cultivated at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere and cells are harvested at various time points in order to monitor time-dependent effects. Cells are spun down and washed with PBS, resuspended in 400  $\mu$ l of RLT buffer (Qiagen RNeasy Total RNA Isolation Kit) with 143 mM  $\beta$ -mercaptoethanol, the DNA is sheared with a 20 g needle for at least 5 times and stored at -70°C.

Total RNAs are isolated with the Qiagen RNeasy Total RNA Isolation Kit (Qiagen) according to the manufacturer's protocol. Purified RNA is digested with RNase-free DNase (Qiagen) and used for TaqMan analysis.

#### TaqMan analysis

Taqman analysis is used to determine mRNA-levels for FPRL-1 in cell lines after treatment with and without various stimuli at different time points. Total RNA isolated from U937 cells that were treated for 3 days with 10 nM retinoic acid is used in order to optimize reaction conditions for determining the mRNA-levels of FPRL-1 and setting standard curves for FPRL-1 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a housekeeping gene. Quantification of FPRL-1 is done with the following primers: Forward primer (rhHM63 668FP, SEQ ID NO:22), Reverse primer (hHM63 525(+))RP, SEQ ID NO:23) and TaqMan probe (rhHM63 629(-))TP, SEQ ID NO:24) labeled with reporter dye FAM at the 5' end and quencher dye TAMRA at the 3' end. The mRNA-levels for GAPDH are determined with a



predeveloped kit for GAPDH "TaqMan GAPDH Control Reagents" (P/N 402869) from PE Applied Biosystems. The GAPDH probe is labeled with JOE as the reporter dye and TAMRA as the quencher dye. RT-PCR reactions are performed with the "TaqMan EZ RT-PCR Core Reagents" (P/N N808-0236) kit from Perkin Elmer. Standard curves for FPRL-1 and GAPDH are performed with increasing concentrations of RNA from U937 cells treated with 1  $\mu$ M retinoic acid ranging from 0, 5, 10, 25, 50 to 100 ng per assay. Reaction mixes contain 1X TaqMan EZ-buffer, 3 mM Mn(Oac)<sub>2</sub>, 300  $\mu$ M dATP, dCTP, dGTP, and 600  $\mu$ M dUTP, 2.5 U rTth DNA polymerase, 0.25 U AmpErase UNG in a total volume of 25  $\mu$ l. For analysis of FPRL-1 reaction mixes include 300 nM of rhHM63 668(-)FP and hHM63 525(+)RP and 100 nM of rhHM63 629(-)TP. The primer concentrations for determining GAPDH levels are 200 nM for each primer and 100 nM for the Taqman probe. In order to determine mRNA levels for FPRL-1 and GAPDH in human subjects and cell lines, 16 to 50 ng RNA per reaction are used. All samples are run in triplicate. The reactions are performed with "MicroAmp Optical 96-well reaction plates" sealed with "MicroAmp Optical Caps" (PE Applied Biosystems) in an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). The PCR conditions are 2 minutes at 50°C, 30 minutes at 60°C, 5 minutes at 95°C, followed by 40 cycles of 20 seconds at 94°C and 1 minute at 59°C. Data analysis is done either by determining the mRNA levels for FPRL-1 and GAPDH according to the standard curves or by directly relating C<sub>T</sub> values for FPRL-1 to C<sub>T</sub> values for GAPDH. The latter procedure can be applied for these genes since the efficiencies for both reactions are in good agreement with each other (around 95%).

Table 15: Expression of FPRL-1 in MonoMac6 cells after stimulation with 10 nM PMA

t (h)	ng FPRL-1 mRNA/ ng GAPDH mRNA
0	0.00
1	0.00
3	0.00
12	0.00
24	0.00
48	0.43
72	0.01

Table 16: Expression of FPRL-1 in MonoMac6 cells after differentiation with 10 nM PMA and stimulation with 20 ng/ml LPS

t (h)	ng FPRL-1 mRNA/ ng GAPDH mRNA
0	0.00
1	0.00
3	0.00
12	1.27
24	2.19
48	2.90
72	1.27

Table 17: Expression of FPRL-1 in MonoMac6 cells after differentiation with 10 nM PMA and stimulation with smoke

t (h)	<b>Fold induction of FPRL-1</b>
0	1.00
1	0.02
3	0.14
6	4.44
12	9.90
25	9.35
48	8.73

Table 18: Expression of FPRL-1 in THP-1 cells after differentiation with PMA and stimulation with LPS

t (h)	Fold induction of FPRL-1
0	1.00
1	0.23
3	1.81
9	15.77
24	0.82
48	1.59

In order to examine the effects of ligands for FPRL-1, MonoMac6 cells are seeded at a density of 250,000 cells/ml in 24-well plates (with 2 ml per well), grown for 24 hours at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere before stimulation with 200 nM lipoxin A4 (Biomol), W-peptide (1 µM) (synthesized by Metabion, Martinsried), and LPS (Sigma) as a positive control. Cells are harvested at different time points, and total RNA is isolated as described above using the Qiagen RNeasy Total RNA Isolation Kit (Qiagen).

The sequence of the W-peptide (Baek et al. 1996, J. Biol. Chem 271, 8170-8175) is W-K-Y-M-V-m.

The RNA is used for Taqman analysis in order to monitor the expression of inflammatory markers like TNFα, IL-8, and MMP-12.

Table 19: Expression of TNFα in MonoMac 6 cells after stimulation with lipoxin A4 and W-peptide

	<b><u>Fold Induction</u></b>	Fold Induction
t (h)	<b><u>Lipoxin A4 (200 nM)</u></b>	W-peptide (1 µM)
0	1.00	1.00
3	2.43	1.03

Table 20: Expression of IL-8 in MonoMac 6 cells after stimulation with lipoxin A4 and W-peptide

	<b><u>Fold Induction</u></b>	Fold Induction
t (h)	<b><u>Lipoxin A4 (200 nM)</u></b>	W-peptide (1 $\mu$ M)
0	1.00	1.00
3	1.99	1.54

Table 21: Expression of MMP-12 in MonoMac 6 cells after stimulation with lipoxin A4 and W-peptide

	<b><u>Fold Induction</u></b>	Fold Induction
t (h)	<b><u>Lipoxin A4 (200 nM)</u></b>	W-peptide (1 $\mu$ M)
0	1.00	1.00
3	1.42	1.51

Since an increased invasion of macrophages in peripheral airways of COPD patients can be observed, we tested the chemotactic ability of MonoMac6 cells which serve as a cell culture model for alveolar macrophages. Chemotaxis of MonoMac6 is determined by administering different ligands for FPRL-1.

MonoMac6 cells are treated with PMA for 24-30 hours in order to induce an activation state of the cells. Cells are harvested, washed twice with RPMI 1640 without supplements, and seeded at a density of 500,000 cells/well (24-well plate) in the presence of 10 nM PMA. After 24-30 hours cells are released from the substratum by repeated rinsing with a pipet, spun down, counted and adjusted to a density of  $1 \times 10^6$  cells/ml of RPMI 1640 media without supplements but in the presence of 10 nM PMA. Chemotaxis is performed in a 48-well chemotaxis chamber (Neuroprobe Inc.) and polycarbonate membranes with a pore size of 8  $\mu$ m (Neuroprobe Inc.). The lower wells of the chamber are filled with 28  $\mu$ l of different concentrations of lipoxin A4, W-peptide, MCP-1 as a positive control, and RPMI 1640 media

without supplements (including 10 nM PMA) as a negative control. The lower wells are covered with the polycarbonate membrane and the upper compartments of the chamber are filled with 50  $\mu$ l of the cell suspension (50,000 cells per well). After 4 hours of migration at 37°C, 5% CO<sub>2</sub>, the cells on the upper part of the membrane are scraped off and the cells attached at the lower part of the membrane are stained with the Diff Quik Staining Set (Dade Behring) according to the manufacturer's protocol. Stained cells are counted in 6 to 8 high power fields at a magnification of 250X with a light microscope. The migration index represents the fold increase in the number of cells migrated in response to the chemoattractant over control medium.

Table 22: Migration of MonoMac6 cells in response to lipoxin A4, W-peptide, and MCP-1

Stimulus	Migration Index
MCP-1 (20 ng/ml)	2.59
Lipoxin A4 (1 $\mu$ M)	1.68
Lipoxin A4 (100 nM)	1.31
Lipoxin A4 (10 nM)	0.86
W-peptide (1 $\mu$ M)	2.46
W-peptide (100 nM)	1.23
W-peptide (10 nM)	0.95

The above examples as well as a cell of each of the above cell culture models are used for determining whether a substance is an inhibitor or an activator of an ILM-receptor of the invention which is deregulated in a macrophage according to the invention by adding a substance to be tested and subsequent measuring of a respective above described effect.